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Cell surface display yields evolvable, clickable antibody fragments

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Abstract

Non-canonical amino acids (ncAAs) provide powerful tools for engineering the chemical and physical properties of proteins. However, introducing ncAAs into proteins can affect protein properties in unpredictable ways, necessitating screening efforts to identify mutants with desirable properties. In this work, we describe an *E. coli* cell surface display platform for evolving clickable antibody fragments. This platform enabled isolation of antibody fragments with improved digoxigenin binding and modest affinity maturation in several different ncAA contexts. Azide-functionalized fragments exhibited improved binding kinetics relative to their methionine counterparts, facile chemical modification via the azide-alkyne cycloaddition, and retention of binding properties after modification. The results described here suggest new possibilities for protein engineering, including modulation of molecular recognition events by ncAAs and direct screening of libraries of chemically modified proteins.

Keywords

non-canonical amino acids; click chemistry; antibodies; directed evolution

The introduction of non-canonical amino acids (ncAAs) into recombinant proteins enables enhancement or alteration of protein function.[1,2] Replacement of canonical amino acids by ncAAs, in response to either sense codons or nonsense codons, can lead to marked changes in reactivity, stability, and other properties.[3–9] In either case, introduction of ncAAs may lead either to loss of protein function or to beneficial changes in behavior. High-throughput screening methods and directed evolution[10,11] can be used to isolate ncAA-modified protein variants with novel and desirable characteristics.

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Supporting information for this article is available on the WWW.

Here we present a cell surface display method for evolving binding proteins that contain reactive, "clickable" ncAAs. Specifically, we replace the methionine (Met; **1**; Figure 1A) residues in single chain variable fragment (scFv) mutants of the anti-digoxigenin 26-10 antibody[12] with the Met surrogates homopropargylglycine (Hpg; **2**), azidohomoalanine (Aha; **3**) or norleucine (Nrl; **4**). The display platform[13,14] enables identification of variants with improved expression levels, solubility, and antigen-binding properties. Several of the isolated Aha variants exhibit improved binding kinetics relative to their Met counterparts, and the azide side chains of Aha can be chemically modified under mild conditions without affecting binding function. Antigen binding and protein modification can be investigated concurrently for scFvs displayed on the *E. coli* cell surface.

We first used flow cytometry to investigate the function of the 26-10 anti-digoxigenin scFv displayed on the surface of *E. coli* cells in the Lpp-OmpA' display system (see Supporting Information for display details).[13,14]ScFv function was assessed in multiple amino acid contexts using BODIPY FL digoxigenin **5** as a probe of binding behavior (Figure 1). Cells displaying the Met form of the scFv could be readily labeled with **5** (Figure 1B), while cells displaying the Hpg form exhibited low fluorescence levels (Figure 1C). Fluorescence levels of cells displaying the Aha-substituted scFv were reduced relative to the cells expressed in Met (Figure 1D), while cells displaying the Nrl-substituted construct exhibited unchanged fluorescence levels (Figure 1E).

The loss of function of the Hpg form of the scFv led us to investigate whether point mutations could enable recovery of digoxigenin binding. We developed an error-prone PCR library (Library 1) in which the antibody fragment and the majority of the display anchor were subjected to mutation. In the first round of sorting, Library 1 was expressed and sorted under conditions leading to partial substitution of Hpg for Met (Figure S1).[15] Sorting this population led to enrichment of clones exhibiting Hpg tolerance. Using expression conditions leading to near-complete replacement of Met, the Hpg-tolerant population was further enriched for functional clones in two additional rounds of sorting (Figure S1).

Individual clones from the thrice-sorted Library 1 were isolated, tested for their ability to bind antigen when expressed in Hpg form on the *E. coli* cell surface, and sequenced. All ten clones bound **5** when Met was replaced with Hpg, and each variant contained at least one amino acid mutation in the scFv (Table S1). While some display-anchor mutations were identified during screening of Library 1 and Library 2 (see below), none appeared to affect scFv properties in any significant way. We performed additional analysis of Mut2, a clone recovered from five of the ten colonies (Figure 1, Table 1, Table S1). In flow cytometry experiments, cells displaying Mut2 bound more **5** than cells displaying the parent 26-10 scFv, irrespective of whether the scFv was expressed in its Met, Hpg, Aha or Nrl forms. The effect was most striking for the Hpg and Aha forms of the protein (Figure 1G, H).

Seeking to improve the binding behavior of the scFv in multiple ncAA contexts, we prepared Library 2 from DNA isolated from thrice-sorted Library 1 via error-prone PCR, and attempted to isolate variants with improved binding after replacing Met with ncAAs. Four rounds of sorting under increasingly stringent conditions were performed, using kinetic competitions in the last two rounds to isolate clones with the lowest off rates (Figure S2,

Table S2).[16] Individual clones were picked after three (Hpg and Aha contexts) and four (Hpg, Aha, and Nrl contexts) rounds of enrichment, sequenced (Table S3), and subjected to on-cell off-rate measurement (Table S4).[16] Most clones exhibited off rates equal to or lower than the off rates of the parent construct or Mut2 when expressed in the same amino acid context.

We examined the sequences of individual clones and the sorted populations in detail (Table 1, Figure 2, Table S5). Mutations at Met codons were common; the Met residues at positions H20 and H80 (Kabat numbering) were replaced in the majority of variants. On the other hand, the Met residues at positions H34 and H100B were conserved. The preservation of M(H100B) is especially striking in view of the fact that it is in direct contact with the antigen.[12] The data also reveal the frequent addition of a Met codon at position H82C; Met replaces a leucine at this position in more than 50% of all sequenced variants. Of the mutations not involving Met, S(H24)F, S(H24)Y, and F(H29)S occurred most frequently. Quantitation of mutational frequencies in the different populations indicated that ncAA context changes mutational frequency only modestly (Table S5).

We produced and characterized several antibody fragments in soluble form. Each scFv of interest was produced in its Met form and in the ncAA form in which it was isolated. In addition, attempts were made to produce the parent 26-10 scFv and Mut2 in Met and all ncAA contexts. The parent construct could be expressed and purified only in its Met and Nrl forms, while Mut2 could be expressed and purified in all four forms. MALDImass spectrometry indicated replacement of 80–93% of Met by the ncAA in each isolated variant (Table S6, S7). Table 1 and Figure 2 summarize the mutations found in the selected scFv variants.

Variation in the antigen-binding behavior of the variants was dominated by changes in k_{off}, the dissociation rate constant. Surprisingly, the kinetic data indicate that replacement of Met by Aha in a given variant consistently decreased k_{off} by a factor of two. Replacement of Met by Hpg increased k_{off} by a factor of four, while replacement of Met by Nrl caused negligible changes in k_{off}. It seems most likely that changes in k_{off} are most sensitive to the identity of the residue at position H100B, which is in direct contact with the antigen.[12] Modest reductions in k_{off} were achieved through further screening in both the Hpg and Nrl contexts, with the Hpg form of Hpg3x3 and the Nrl form of Nrl4x11 exhibiting two-fold lower dissociation rate constants as compared to Mut2 in the same ncAA contexts.

The Aha and Met forms of several scFv variants were subjected to strain-promoted azide-alkyne cycloaddition (SPAAC) with dibenzocyclooctyne dye **6** (Figure 3A, Figure S3). In-gel fluorescence experiments confirmed selective labeling of the Aha forms; minimal background labeling of the Met forms was detected. Mass spectrometry and the lower reactivity of the Aha form of Aha4x4 (which lacks Aha at H34) implicated position H34 as a major site of modification (Figure S4, Table S8). Our attempts to modify Hpg- and Aha-containing scFvs by the copper-catalyzed azide-alkyne cycloaddition were unsuccessful, possibly because of the limited surface accessibility of the reactive ncAA side chains in the proteins (Figure S5).[17]

SPAAC-modified scFv fragments retain antigen-binding behavior, as shown by western blotting assays. Samples for western blotting were prepared by adding digoxigenin-modified bovine serum albumin (BSA) in varying amounts to *E. coli* cell lysates. The samples were transferred to nitrocellulose membranes, and the membranes were probed with the Aha or Met forms of Aha4x5 fragments previously treated with **6**. Scanning the membranes for fluorescence emission from **6** (Figure 3B) revealed that only the Aha form of the protein enabled detection of digoxigenin-modified BSA. To confirm that treatment with **6** did not adversely affect the Met form of the protein, the blots were further probed for the hexahistidine tags on the scFvs (Figure 3C). These experiments confirmed that both forms of Aha4x5 remained capable of binding to digoxigenin after SPAAC. Biacore assays further indicate that SPAAC conditions did not seriously alter antibody kinetic properties; k_{on} values were reduced slightly, while k_{off} values remained unchanged (Table S9).

We extended our functional analysis of tagged conjugates to scFvs displayed on the *E. coli* cell surface. We subjected *E. coli* cells displaying the Aha or Met forms of Aha4x5 to 16-hour labeling reactions with **7** (Figure 3D).[18,19] We then labeled the cells with **5** and streptavidin-phycoerythrin and performed flow cytometry. Cells displaying the Aha form of the scFv exhibited substantial fluorescence in both channels (Figure 3E), confirming that the Aha form of Aha4x5 retains binding function after chemical modification on the cell surface, while cells displaying the Met form bind **5** but exhibit low reactivity toward the cyclooctyne (Figure 3F). Additional experiments indicated that treatment of cells with unlabeled digoxin prior to treatment with **5** blocks the binding of **5** to scFvs regardless of chemical modification state (Figure S6). These experiments also revealed that some binding activity appears to be lost upon treatment of cells displaying the Aha form of Aha4x5 with **7**; these losses in binding function may reflect an increase in crowding and steric hindrance on the surface of cells after treatment of **7** or denaturation of some scFvs during the click reaction. Finally, experiments verified that **7** reacts primarily with Aha side chains in scFvs, not Aha in other newly synthesized proteins; fluorescence values suggest that approximately 75 percent of **7** detected on the cell surface has reacted with displayed scFvs, with the remaining 25 percent reacting with other membrane proteins.

Having shown that both antigen-binding and modification of displayed scFvs can be monitored in a single flow cytometry experiment, we used a subset of samples to demonstrate the separation of various cellular populations via fluorescence-activated cell sorting (FACS). We mixed together cells exhibiting antigen binding only, chemical modification only, simultaneous binding and modification, and neither binding nor modification (Figure 4A). Four-way sorting of this mixture resulted in the recovery of clean populations exhibiting all combinations of binding and modification (Figure 4B–E). These results suggest that this display platform should be amenable to simultaneous, high throughput evaluation and isolation of proteins based on chemical reactivity and binding function.

This work demonstrates how high-throughput screening of antibody fragments containing ncAAs can yield proteins with novel properties. The screening methods described here yielded scFvs with enhanced expression and antigen-binding behavior. Fragments containing Aha exhibited improved dissociation rate constants and susceptibility to chemical

modification under mild conditions. These properties were demonstrated both in solution and on the *E. coli* cell surface. Our approach is complementary to the screening of libraries of antibody fragments via phage display with ncAAs[20] and to the introduction of reactive amino acids into antibody fragments site-specifically.[21] In the future, incorporating multiple ncAAs into recombinant proteins may enable re-design of antibody complementarity determining regions[22] with unconventional side chains to enhance molecular recognition. Cell surface-display of ncAA-modified proteins should also enable direct screening of libraries of chemically modified proteins of interest in the engineering of bi- or multivalent proteins,[23] switchable sensors,[24] or cyclized peptides.[25]

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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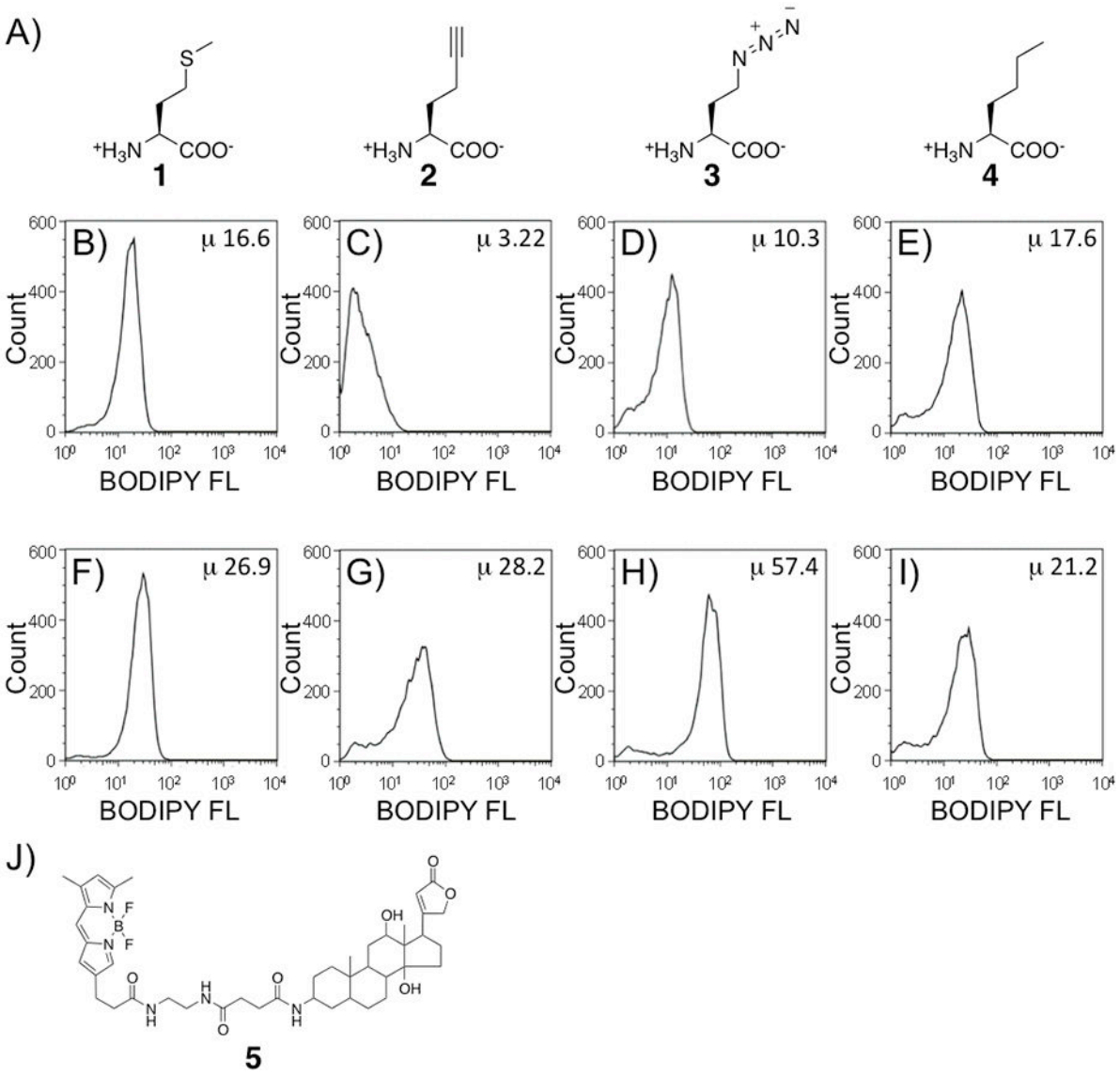

Figure 1.

Flow cytometry studies of the binding properties of cell surface-displayed scFvs. A) Amino acids used in study. B–E) Parent 26-10 expression in media supplemented with Met (**1**; B), Hpg (**2**; C), Aha (**3**; D), and Nrl (**4**; E), followed by labeling of cells with BODIPY FL digoxigenin (**5**; J) and flow cytometry. F–I) Mut2 expression in **1** (F), **2** (G), **3** (H), and **4** (I), followed by labeling of cells with **5** and flow cytometry. J) Chemical structure of **5**. μ, mean fluorescence intensity.

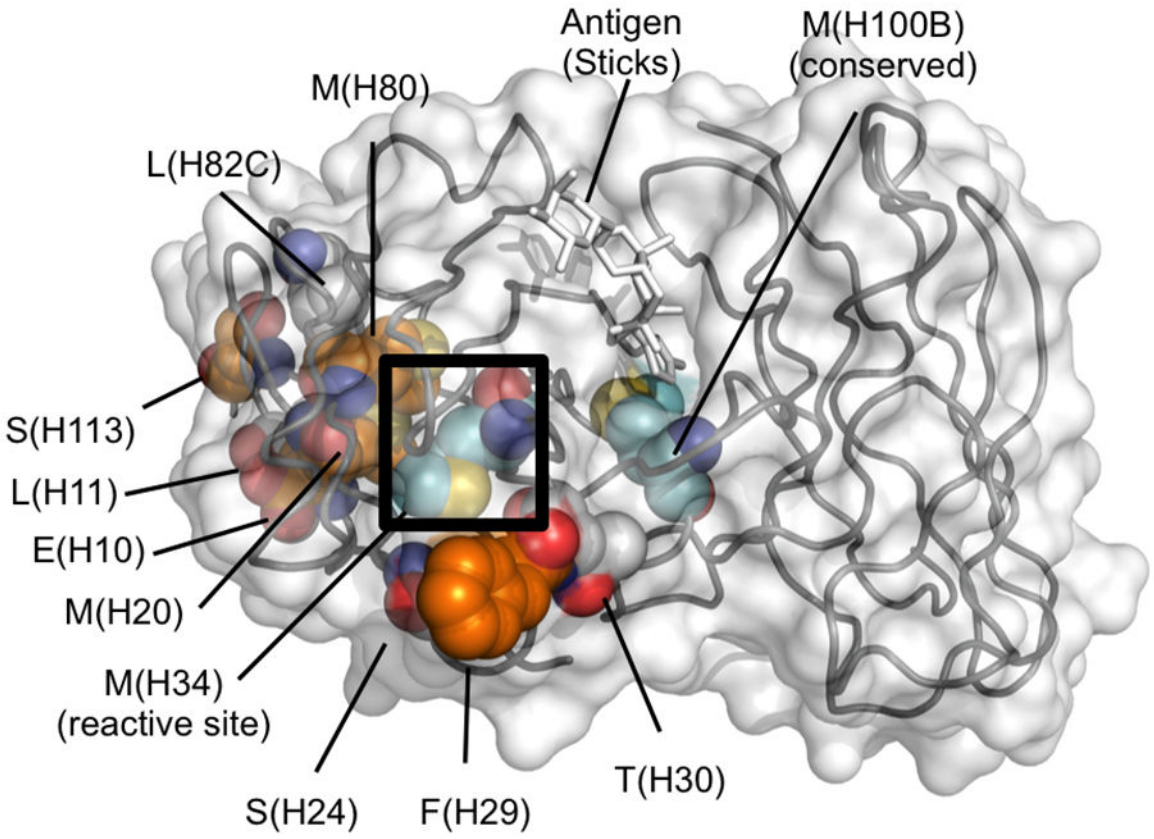

Figure 2.

Amino acids mutated in 26-10 scFv variant Aha4x5 (space filling, colored by atom, carbons in orange), amino acids mutated in other scFvs shown in Table 1 (space filling, colored by atom, carbons in gray), and conserved methionines (space filling, colored by atom, carbons in cyan). The methionine at position H34 is boxed as the likely site of chemical modification. Element colorings: carbon, orange, gray, or cyan; oxygen, red; nitrogen, blue; sulfur, yellow. Structure produced from the “A” and “B” chains of PDB structure 1IGJ[12] with MacPyMOL.

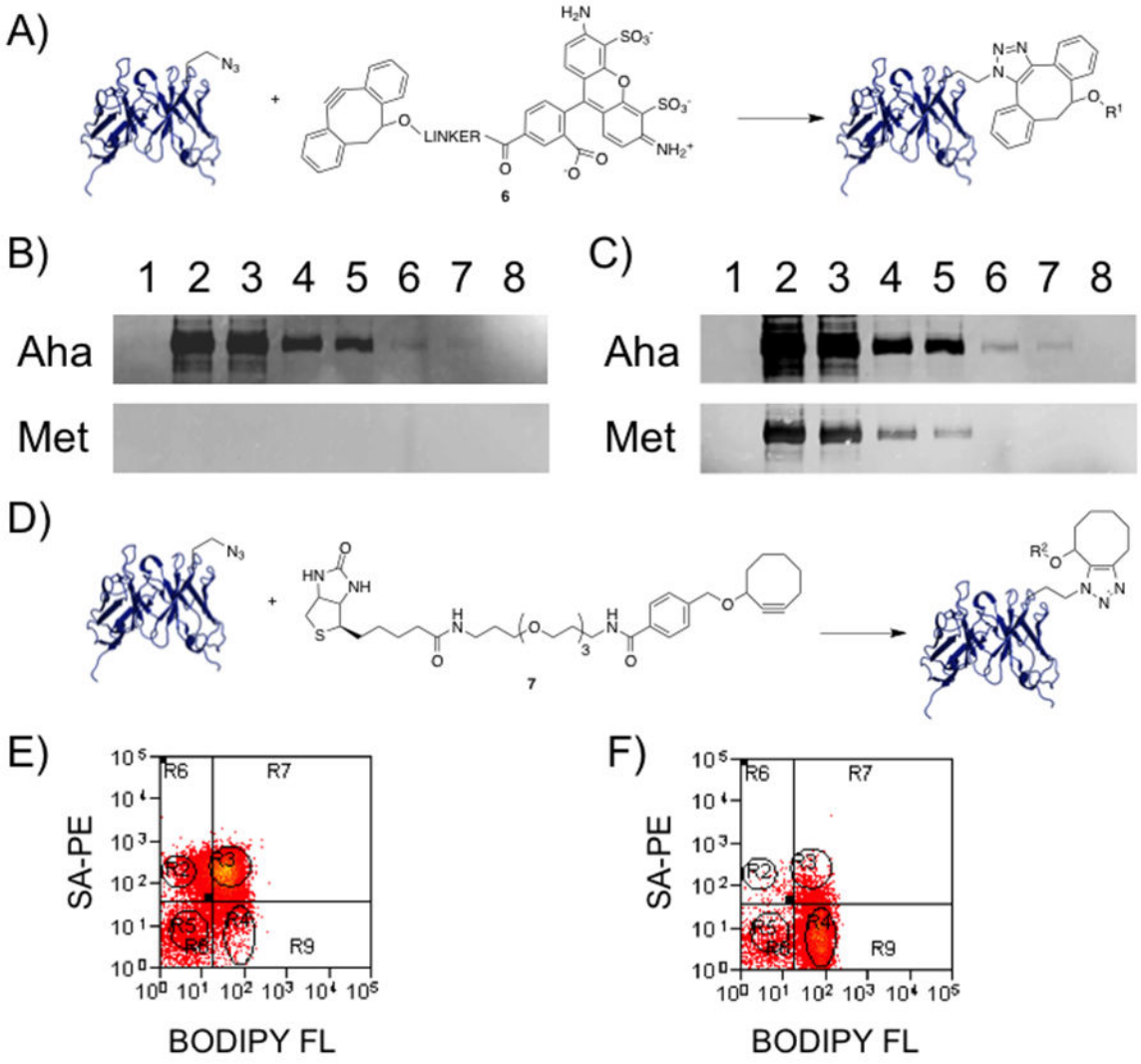

Figure 3.

Strain-promoted click chemistry, western blotting, and flow cytometry with Aha-containing scFvs. A) Reaction of Aha-containing scFvs with Alexa Fluor 488 dibenzocyclooctyne **6**. The structure of the linker has not been disclosed by the manufacturer. R^1: Alexa Fluor 488-LINKER.B) Western blots for the detection of digoxigenin conjugated to bovine serum albumin (BSA-dig). Duplicate blots were probed with the Met and Aha forms of Aha4x5 after the scFvs were subjected to SPAAC. Each lane of the SDS-PAGE gel was loaded with ~5 μg *E. coli* lysate and the following protein samples: Lane 1, 1000 ng BSA (unlabeled). Lanes 2–8: 1000, 500, 100, 50, 10, 5, and 1 ng BSA-dig, respectively. C) The blots were further probed for hexahistidine purification tags present in the scFvs in order to ensure that SPAAC reactions did not adversely affect antigen binding. A secondary anti-penta-His antibody labeled with Alexa Fluor 647 was used for detection. Lanes same as in (B). D) Reaction of Aha-containing scFvs with biotin-cyclooctyne **7**. R^2: biotin-PEO. E, F) Flow cytometry with the Aha (E) and Met (F) forms of Aha4x5 displayed on the *E. coli* surface.

Cells were treated with **7** for a period of 16 hours and then labeled with **5** and streptavidin-phycoerythrin (SA-PE) to detect antigen binding and chemical modification, respectively.

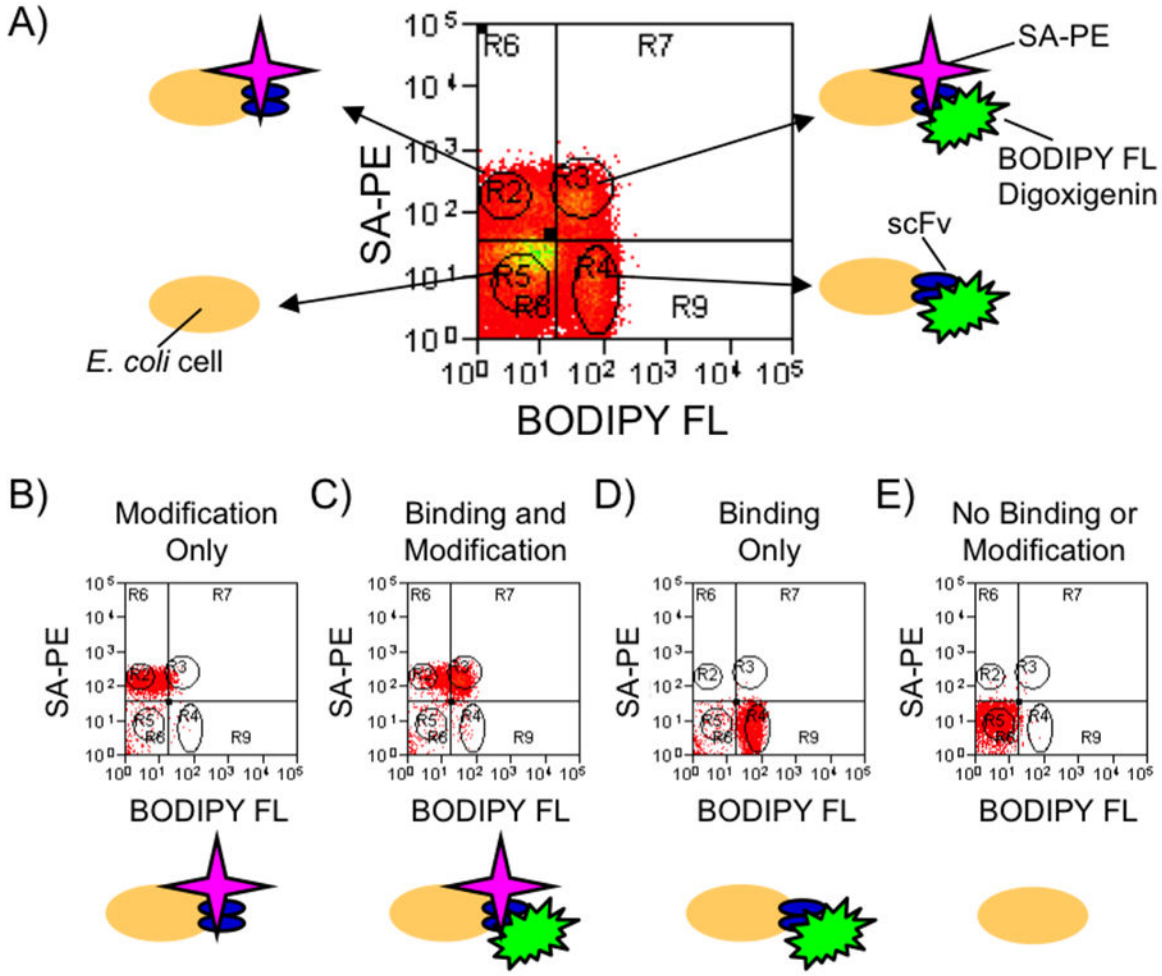

Figure 4.

Fluorescence-activated cell sorting for the isolation of functional, modified proteins. A) Two-dimensional dot plot of a mixture of four populations of cells: Aha4x5 expressed in Met (binding but no chemical modification), Aha4x5 expressed in Aha (binding and modification), Aha4x5 expressed in Aha blocked with nonfluorescent digoxin (chemical modification but no binding), and Aha4x5 grown in Aha without induction (no chemical modification or binding). Each cellular population was treated with **7** and exposed to streptavidin-phycoerythrin (SA-PE) and **5** for detection of chemical modification and binding, respectively. B–E) Sorted populations of cells from the gated regions R2–R5 of (A) led to the isolation of populations with distinct binding and chemical modification properties.

Table 1

Amino acid mutations and dissociation kinetics of selected scFvs in Met and ncAA forms.

Clone	Amino Acid Mutations in scFv									k_{off} (10^{-3} s^{-1}) in Amino Acid Context			
	E (H10)	L (H11)	M (H20)	S (H24)	F (H29)	T (H30)	M (H80)	L (H82C)	S (H113)	Met	Aha	Hpg	Nrl
Parent	-	-	-	-	-	-	-	-	-	1.85 ± 0.15			2.31 ± 0.39
Mut2	-	-	I	-	-	-	L	M	-	1.72 ± 0.17	0.96 ± 0.16	8.09 ± 0.53	2.07 ± 0.20
Aha4x5[a]	A	-	I	-	S	-	L	-	-	1.31 ± 0.29	0.68 ± 0.11		
Hpg3x3	-	S	I	F	-	S	L	M	-	0.93 ± 0.07		4.63 ± 0.34	
Nrl4x11	-	-	L	Y	S	-	L	-	F	0.93 ± 0.09			1.08 ± 0.07

[a] Aha4x5 also contains a G to V mutation at position nine of the $(Gly_4Ser)_3$ linker.